OVEREXPRESSION IN YEAST AND PLANTS OF A GENE ENCODING GLYCEROL 3-PHOSPHATE ACYLTRANSFERASE

TECHNICAL FIELD

The invention relates to the production of fats and oils for commercial and industrial uses. More particularly, the invention relates to a process by which natural oil or fat levels in organisms may be increased, fatty acid composition of triacylglycerides may be altered, and to nucleotide sequences which may be introduced into organisms to cause the increase, and plasmids, vectors, etc. useful in the process.

BACKGROUND ART

Non-animal-source oils are produced mainly for edible purposes but their use in non-edible applications is expected to increase due to declining fossil fuel supply [1](Kinney, 1998). More than 65 million metric tons of vegetable oils are produced currently with a total value of $US 25 billion [2,3](Browse et al., 1998; Murphy, 1999). World demand for vegetable oils has increased by 300% since 1960. Further, the market share of animal-derived fats has declined from 39% of the total in 1960 to 26% in 1990. All of these factors have contributed to a demand by industry for higher vegetable oil content in plant seeds in order to be cost effective during production and processing[4] (Bright and Hawkes, 1998). Biotechnology offers avenues for meeting this demand through identification and manipulation of the biochemical pathways that lead to oil production.

Glycerol-3-phosphate acyltransferase (GPAT) catalyses the first reaction in triacylglyceride synthesis via the Kennedy pathway. It uses glycerol-3-phosphate (G-3-P) and acyl-coenzyme A (acyl-CoA) thioesters to synthesise lysophosphatidic acid (LPA). In fat storing organs e.g., seeds and adipose tissues, the remaining reactions are catalysed by a *lyso*phosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphatase (PAPase) and diacylglycerol acyltransferase (DAGAT). Thus far, LPAATs and DAGATs have been the foci of studies on TAG biosynthesis. The role of GPAT has received less attention. Several studies have shown that PAPase controls the rate limiting step of TAG biosynthesis in mammals

and yeast and DAGAT is the rate limiting enzyme in plants [1](Perry and Harwood, 1994).

Only a limited number of genes have been reported to enhance oil content (for example, Zou et al.[2], 1997, Zou et al.[3], 1998). There is a need to explore other approaches using other genes which might be more effective alone or in combination with previously cloned genes.

A plastidial GPAT gene has been used by others to change the fatty acid composition of membrane lipids and to improve chilling tolerance (Murata et al.[4], 1992 and Nishizawa[5], 1996). The bacterial GPAT gene *(plsB)* has also been used to change the fatty acid composition of membrane lipids and to decrease chilling tolerance (Wolter et al.[6], 1992). No study has yet used GPAT genes to increase oil content in an organism.

In an article entitled "Engineering and breeding of new oil crops – Aclytransferases from basic science to modified seed oils", Fett/Lipid 100 (1998), Nr. 4-5, S. 161-166, Margrit Frentzen discloses that different discrete acyltransferases are involved in glycerlipid biosynthesis in plant cells. Specifically, chimeric 1-acylglycerol-3-phosphate acyltransferase genes have been successfully utilized to achieve the synthesis of rapeseed oil with homogeneous fatty acid distributions desired for industrial applications. Moreover, it is mentioned that evidence has been provided that expression of acyltransferase genes in transgenic rapeseed plants can improve not only oil quality but also oil yield.

PCT patent application WO 92/13082, which was filed by Kirin Beer KK and was published on August 6, 1992, discloses the genetic engineering of higher plants to confer chilling resistance. This involves producing a plant containing more than normal unsaturated fatty acids in membrane lipids. A preferred embodiment of such a plant is a transgenic plant expressing a polypeptide with a glycerol 3-phosphate acyltransferase activity that has a higher substrate selectivity for oleoyl-ACP than for palmitoyl-ACP. Specifically, an Atase gene from *A. thaliana* was expressed in transgenic tobacco plants.

DISCLOSURE OF THE INVENTION

It is an object of the invention to provide a method for increasing the oil content of an organism.

It is a further object of the invention to provide a genetically altered organism with enhanced GPAT activity, relative to the wild type.

It is a further object of the invention to produce an organism having increased oil or fat production capability, relative to the wild type.

Another object of the invention is to produce DNA clones, constructs and vectors suitable for modifying the genomes of organisms to increase the production of triacylglycerides (TAGs), relative to the wild type.

An additional object of the invention is to produce an organism having an altered fatty acid composition in its triacylglycerides, relative to the wild type.

Still a further object of the invention is to identify, isolate and clone a genetic element that may be used to modify the natural formation of triacylglycerols in plants

in order to increase the yield of commercial plant oils, or to modify their composition to achieve specific commercial improvements of plants and plant products.

In a first aspect, the invention provides a method for increasing the oil content of an organism by inserting in the organism a DNA encoding a protein having glycerol 3-phosphate acyltransferase activity.

In a second aspect, the invention provides an organism transformed with a DNA, wherein the DNA encodes a protein having GPAT activity, and the organism, after transforming, has enhanced ability to produce triacylglycerides.

In a third aspect, the invention provides a vector for genetically transforming an organism, wherein the vector comprises a DNA encoding a protein having GPAT activity, and the organism, after transforming, exhibits enhanced production of triacylglycerides.

In a fourth aspect, the invention provides a method for modifying the fatty acid composition of triacylglycerides produced by an organism, wherein the organism is transformed with a DNA encoding a protein having GPAT activity.

All organisms have within their genome a gene encoding a protein having GPAT activity. The invention relates to a method for expressing in an organism at least one additional DNA sequence encoding a protein having GPAT activity.

The inventors chose to target GPAT because of its role in the Kennedy pathway, a pathway that is common to all organisms.

In a preferred embodiment, the invention pertains to micro-organisms. In a particularly preferred embodiment, to yeasts and plants.

The method of the invention is particularly suited to the production of oil seed plants having enhanced TAG content, or having modified fatty acid composition in their TAGs. The terms "seed oil plant" and "oil seed crop" are meant to encompass any plant or crop from which the oil may be isolated in marketable quantity. Some plants or crops having TAGs with particularly interesting fatty acid composition are grown

for the production of TAGs, even though the lipid content is low (e.g. less than 1 wt%). The method of the invention may be used in such plants to increase the content of TAG. Preferred plants or crops are those having a seed lipid content of at least 1 wt% (in the wildtype). Some illustrative examples of oil seed crops are as follows (trivial names are given in parentheses):

Borago officinalis (Borage); *Brassica* species, for example mustards, canola, rape, *B. campestris*, *B. napus*, *B. rapa*; *Cannabis sativa* (Hemp, widely uses as a vegetable oil in Asia); *Carthamus tinctorius* (Safflower); *Cocos nucifera* (Coconut); *Crambe abyssinica* (Crambe); *Cuphea* species (*Cuphea* produce medium chain fatty acids of industrial interest); *Elaeis guinensis* (African oil palm); *Elaeis oleifera* (American oil palm); *Glycine max* (Soybean); *Gossypium hiristum* (Cotton – American); *Gossypium barbadense* (Cotton – Egyptian); *Gossypium herbaceum* (Cotton – Asiatic); *Helianthus annus* (Sunflower); *Linum usitatissimum* (Linseed or flax); *Oenethera biennis* (Evening primrose); *Olea europea* (Olive); *Oryza sativa* (Rice); *Ricinus communis* (Castor); *Sesamum indicum* (Sesame); *Soja max* (Soybean – note Glycine max is the major species); *Triticum* species (Wheat); and *Zea maize* (Corn).

Three types of plant GPAT have been reported: plastidial (P), mitochondrial (M), and cytosolic (ER). They exhibit different specificities towards acyl-ACP and acyl-CoA derivatives of fatty acids [11](Frentzen, 1993). The P-GPAT is mainly concerned with phospholipid biosynthesis in chloroplasts and has been shown to use both acyl-ACPs and acyl-CoAs as substrates, although the latter with a lower efficiency[12] (Wilkinson and Bell, 1997). The ER form of GPAT is the most important for TAG biosynthesis but a plant gene has not yet been cloned. The ER-GPAT uses acyl-CoAs. The GPAT from the enteric bacterium *Escherischia coli* can use both acyl-ACP and acyl-CoA equally well[13](Wilkinson and Bell, 1997).

As will be appreciated by persons skilled in the art, the invention also relates to substantially homologous DNA sequences from plants encoding proteins with deduced amino acid sequences of 25% or greater identity, and 40% or greater similarity, isolated and/or characterized and/or designed by known methods using the sequence information of SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4 or SEQ ID NO:5, and to parts of reduced length that are still able to function as

inhibitors of gene expression by use in an anti-sense, co-suppression (Transwitch; [14]Jorgensen and Napoli 1994) or other gene silencing technologies. It will be appreciated by persons skilled in the art that small changes in the identities of nucleotides in a specific gene sequence may result in reduced or enhanced effectiveness of the genes and that, in some applications (e.g. anti-sense or co-suppression), partial sequences often work as effectively as full length versions. The ways in which the gene sequence can be varied or shortened are well known to persons skilled in the art, as are ways of testing the effectiveness of the altered genes. All such variations of the genes are therefore claimed as part of the present invention.

Other preferred degrees of identity to the indicated sequences for both DNA and protein sequences are at least 30%, 40%, 50%, 60%, 70%, 80%, 90% and 95%; and other preferred degrees of similarity are at least 50%, 60%, 70%, 80%, 90% and 95%. The inventors have used a computer program known as MegAlign®, DNASTAR® of DNASTAR Inc., 1228 South Park Street, Madison, WI 53715, USA, for assessing homology. This program is based on the Clustal V algorithm (Higgins and Sharp, 1998): A package for performing multiple sequence alignment on a microcomputer; GENE 73:237-244). For each gap introduced in the alignment, the program deducts a penalty from the score. A higher gap penalty suppresses gapping; a lower value promotes it. The program also assesses penalties based on the length of the gap. The more residues the gap spans, the greater the penalty. The program deducts these penalties from the overall score of the alignment.

When considering altered oil contents or compositions, results from averages of statistically-significant numbers of plants or seeds according to the invention are best compared with results from averages of statistically-significant numbers of untransformed (wild-type) plants or seeds of the same genotype grown under identical conditions at the same time. This allows for the variability of individual plants of the same genotype, particularly when such plants are grown under different conditions. The actual number of plants or seeds used to form the required average may vary, but should be enough to provide a generally constant average whenever such number is selected. Generally, the number should be at least 10, and is more preferably at least 20, 30, 50 or 100.

Alternatively, the oil contents or compositions may be compared with plants of the same species transformed with an open vector (the same vector as that used for the introduction of the DNA of the invention, but with such DNA omitted), grown under identical conditions at the same time. Again, an average of results from a number of such plants, as well as plants transformed according to the present invention, is preferred (the numbers being the same as those indicated above).

The GPAT of the current invention is useful in manipulating GPAT activity, and triacylglycerol bioassembly in plants. For example, by transforming plants with a construct containing the GPAT gene in a sense orientation, possibly under the control of a tissue-specific promoter, the expression of GPAT and accumulation of seed oil can be enhanced or the acyl composition of the seed oil altered. Yet another example would be to express the GPAT cDNA under the control of a constitutive promoter (e.g. 35S ; [15]Datla et al., 1993) to increase the TAG content of vegetative tissues (leaves, roots, stems). This may have particular advantages for altering the starch/oil ratio in root crops.

Alternatively, GPAT expression can be silenced to some degree by anti-sense or co-suppression (Transwitch) phenomena ([16]De Lange et al., 1995; [17]Mol et al., 1990; [18]Jorgensen and Napoli, 1994; [19]Kinney, 1995; [20]Vaucheret et al, 1998; [21]Taylor, 1998). For example, silencing GPAT in a seed specific manner may result in a reduction in TAG accumulation. This could have applications, for example, in reducing the oil content in seed barley to enhance stability during storage. As a second example, seed-specific silencing may lead to a relatively high accumulation of DAG or an increase in the DAG/TAG ratio in the developing or mature seed.

Some of the manipulations and deliverables which are possible using the GPAT gene or a part thereof, include, but are not limited to, the following: seeds with increased or decreased oil content; seeds containing oils with an enhanced diacylglycerol content, seed oils with an altered acyl composition; plants producing larger or heavier seeds; plants exhibiting an enhanced or altered capacity to accumulate storage compounds in other storage organs (e.g. tubers, roots).

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention are illustrated with the help of the drawing:

Figure 1 shows the Seed oil content of wild type (Wt) *Arabidopsis thaliana*, and *A. thaliana* transformed with: vector only (pHS737); the unmodified GPAT encoding DNA from safflower (ctpGPA); the GPAT encoding DNA from safflower from which the transit peptide has been deleted (ctpGPA-TP); the GPAT encoding DNA from safflower from which the transit peptide has been deleted and the ER retention signal has been added (ctpGPA+ERRS); the GPAT encoding DNA from *Escherichia coli* (plsB); and the GPAT encoding DNA from *Escherichia coli* to which the ER retention signal has been added (plsB+ERRS).

BEST MODES FOR CARRYING OUT THE INVENTION

The inventors chose to use the well-accepted model plant system Arabidopsis thaliana for the cloning of GPAT, as a host system for genetic engineering to alter GPAT expression, and to study the effects of altering GPAT expression on seed triacylglycerol bioassembly. Over the past several years, Arabidopsis thaliana, a typical flowering plant, has gained increasing popularity as a model system for the study of plant biology. As a result of the ease with which this plant lends itself to work in both classical and molecular genetics, Arabidopsis has come to be widely used as a model organism in plant molecular genetics, development, physiology and biochemistry ([22]Meyerowitz and Chang, 1985; [23]Meyerowitz, 1987; [24]Goodman et al., 1995). This model dicotyledonous plant is also closely related to Brassica crop species and it is increasingly apparent that information concerning the genetic control of basic biological processes in Arabidopsis will be transferable to other species ([25]Lagercrantz et al., 1996).

Indeed, there are numerous examples wherein studies of the molecular biology and biochemistry of a particular metabolic pathway or developmental process and the possibility of genetically engineering a plant to bring about changes to said metabolic pathway or process, has first been tested in the model plant Arabidopsis, and then shown to yield similar phenotypes in other plants, particularly crop plants.

For example, the extra- plastidial membrane associated oleate (18:1) Δ12 (ω-6) desaturase gene, FAD2, was originally studied and eventually cloned from Arabidopsis thaliana, by identifying the lesion found in an A. thaliana mutant defective in desaturating oleate to produce linoleate (18:2) on the phosphatidylcholine backbone. This resulted in a high oleic acid phenotype in the A. thaliana seed oil ([26]Okuley et al., 1994). Genetic engineering of both soybean (*Glycine max.*) and canola *B. napus* to silence the indigenous FAD2 gene(s) in a seed-specific manner by anti-sense or co-suppression approaches, resulted in similar high oleic acid seed oil phenotypes ([27]Kinney, 1995; 1997).

Transgenic expression of a yeast sn-2 acyltransferase (SLC1-1) gene to achieve modified seed oil content and enhance very long-chain fatty acid content was first performed in Arabidopsis and later shown to yield similar phenotypes in transgenic rapeseed (B. napus) experiments ([28]Zou et al., 1997). Arabidopsis thaliana has repeatedly shown itself to be a useful model system for metabolic engineering of metabolic pathways (e.g. lipid biosynthesis, photosynthesis) or processes (organogenesis, reproductive development etc.) common to all higher plants.

In the area of secondary metabolism/signal transduction, an anthocyanin pathway-specific transcriptional activator from the monocot maize designated as R (the myc transcription factor involved in activation of biosynthetic genes for anthocyanin production in the aleurone cells of maize kernels), was expressed in the dicot Arabidopsis, causing augmented anthocyanin pigmentation in the infloresecences. Subsequent expression in another dicot, tobacco (Nicotiana tabacum), resulted in similar floral pigmentation changes ([29]Lloyd et al., 1992). These experiments demonstrate that whole pathways common to all flowering plants can be co-ordinately controlled through the introduction of transcriptional regulators, and that the mechanisms are common to diverse plant species.

In the context of the current invention, all plant seeds accumulate some triacylglycerol (oil) and this ubiquitous process is affected, at least in part, by the activity of GPAT, as explained previously. Thus, many of the effects observed

following genetic engineering to modulate GPAT expression in Arabidopsis can be expected to result in similar phenotypes when carried out in all other plants.

There are a number of ways by which genes and gene constructs can be introduced into plants, and a combination of plant transformation and tissue culture techniques have been successfully integrated into effective strategies for creating transgenic crop plants. These methods, which can be used in the present invention, have been extensively reviewed elsewhere ([30]Potrykus, 1991; [31]Vasil, 1994; [32]Walden and Wingender, 1995; [33]Songstad et al., 1995), and are well known to persons skilled in the art. For example, one skilled in the art will certainly be aware that, in addition to Agrobacterium-mediated transformation of Arabidopsis by vacuum infiltration ([34]Bechtold et al., 1993) or wound inoculation ([35]Katavic et al., 1994), it is equally possible to transform other plant and crop species, using Agrobacterium Ti-plasmid-mediated transformation (e.g. hypocotyl; [36]DeBlock et al., 1989) or cotyledonary petiole ([37]Moloney et al, 1989) wound infection), particle bombardment/biolistic methods ([38]Sanford et al., 1987; [39]Nehra et al., 1994; [40]Becker et al., 1994) or polyethylene glycol-assisted protoplast transformation ([41]Rhodes et al., 1988; [42]Shimamoto et al., 1989) methods.

As will also be apparent to persons skilled in the art, and as extensively reviewed elsewhere ([43]Meyer, 1995; [44]Datla et al., 1997), it is possible to utilize plant promoters to direct any intended up- or down-regulation of transgene expression using constitutive promoters (e.g. those based on CaMV35S), or by using promoters which can target gene expression to particular cells, tissues (e.g. napin promoter for expression of transgenes in developing seed cotyledons), organs (e.g. roots), to a particular developmental stage, or in response to a particular external stimulus (e.g. heat shock).

Particularly preferred plants for modification according to the present invention include Arabidopsis thaliana, borage (Borago spp.), Canola, castor (Ricinus communis), cocoa bean (Theobroma cacao), corn (Zea mays), cotton (Gossypium spp), Crambe spp., Cuphea spp., flax (Linum spp.), Lesquerella and Limnanthes spp., Linola, nasturtium (Tropaeolum spp.), Oenothera spp., olive (Olea spp.), palm (Elaeis spp.), peanut (Arachis spp.), rapeseed, safflower (Carthamus spp.), soybean

(Glycine and Soja spp.), sunflower (Helianthus spp.), tobacco (Nicotiana spp.), Vernonia spp., wheat (Triticum spp.), barley (Hordeum spp.), rice (Oryza spp.), oat (Avena spp.) sorghum (Sorghum spp.), rye (Secale spp.) or other members of the Gramineae.

The present invention is particularly useful when used to modify the yield or composition of oilseed produced from oilseed crops. Oilseed crops are plant species that are capable of generating edible or industrially useful oils in commercially significant yields, and include many of the plant species listed above. Such oilseed crops are well known to persons skilled in the art.

Once a transgenic oilseed plant has been produced according to the present invention, it can be grown and harvested in conventional ways. Oil may be extracted from harvested seed by collecting and crushing the seed, and/or by methods of solvent extraction, in which the crushed seeds are contacted with a solvent for the oil and the resulting solution is filtered off or decanted and the solvent removed. Other conventional and traditional methods of oil extraction may be used, if desired.

The method of the invention encompasses the transformation of any organism with a DNA encoding a protein having GPAT activity.

As an example of the method of the invention, the inventors have demonstrated the role of GPAT in regulating the amount of TAG by expressing, in yeast and in the plant *Arabidopsis thaliana*, a P-GPAT gene (*ctpgpat*) from safflower [45](Bhella and MacKenzie, 1994) and the GPAT gene (*plsB*) from *E. coli*.

Normally, plastidial proteins encoded by nuclear genes are targeted to plastids by a transit peptide (tp). Removal of the tp will confine such proteins to the cytosol. Since the enzymes of TAG biosynthesis are present in the endoplasmic reticulum (ER) and TAGs are synthesised at the ER, an ER retention signal [46](*errs*; Jackson et al., 1990) which has been shown to target many heterologous proteins including *E.coli* LPAAT to the ER (Weier et al.[47], 1998) was used to target P-GPAT without a tp. The *plsB* gene was used as such and also with an added *errs* sequence.

It is generally accepted in the art that proteins having 60% or greater sequence homology will have identical functionalities. Nonetheless, many cases are known in which far lower sequence homologies (e.g. 25 to 30%) exist and yet the proteins have identical functionalities.

The particular genes mentioned above will have numerous homologous variants, by virtue of the degeneracy of the genetic code. The specific examples described to illustrate the invention obviously relate as well to such homologous variants.

The use of heterologous genes (those GPATs taken from other species) may be particularly advantageous, because the encoded proteins may not be subject to regulation (such as feedback inhibition, or inhibition by native inhibitors) in the same way as the native GPAT.

The inventors used the vector pYES2 (Invitrogen) for transformation of yeast, and the vector pHS737, for transformation of *Arabidopsis thaliana*. Examples of other vectors are:

Yeast vectors:

pYeDP60 (Urban P, Cullin C, Pompon D 1991. Maximizing the expression of mammalian cytochrome P-450 monooxygenase activities in yeast cells. Biochimie 72: 463-472); pCGS109 (Botstein D, David RW, Fink GR, Taunton-Rigby A, Knowlton RG, Mao J-I, Moir DT, Goff CG 1987. GAL 1 yeast promoter linked to non galactokinase gene. US patent No. 4661454); pYEUra3 (Clontech).

Plant vectors:

pHS737 and pHS738 (Selvaraj and Hirji; unpublished); pRD400 (Datla RS, Hammerlindl JK, Panchuk B, Pelcher LE, Keller W. 1992. Modified binary plant transformation vectors with the wild-type gene encoding NPTII. Gene 122:383-384.); pBin19 (Frisch DA, Harris-Haller LW, Yokubaitis NT, Thomas TL, Hardin SH, Hall TC. 1995. Complete sequence of the binary vector Bin19. Plant Mol Biol 27:405-409.); pCGN3223 (Roesler K, Shintani D, Savage L, Boddupalli S, Ohlrogge

JB (1997) Targeting of the Arabidopsis homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. Plant Physiol 113: 75-81).

MATERIALS AND METHODS

Preparation of Chimeric Genes and Expression Vectors.

An open reading frame (*orf*; ~1.1 kb) without *tp* was amplified by PCR from the *ctpgpat* cDNA. Another chimeric gene containing an *errs* at its 3' end was also PCR amplified. The *orf* of the *E.coli plsB* gene (~2.5 kb) was PCR amplified from bacterial DNA without modification or with an *errs* at its 3' end. The blunt-end PCR fragments generated using *Pfu* DNA polymerase were cloned into pSK II (Strategene) cloning vector and were sequenced to confirm the nucleotide sequence as well as incorporation of restriction sites and *errs* sequences into the chimeric genes. The modified genes were labelled as *ctpgpat-tp*, *ctpgpat-tp+errs*, *plsB* and *plsB+errs*. The intact *ctpgpat* cDNA was also used (Bhella and MacKenzie[48], 1994). The sequences of the modified and unmodified genes are shown in SEQ. ID. NOS. 1 to 5.

Transformation of a Yeast

For the yeast expression study, the *ctpgpat* or *plsB* chimeric genes were retrieved as *Bam*HI or *Bgl*II segments, respectively, and were cloned into the *Bam*HI site of the yeast expression vector, pYES2 (Invitrogen), under the transcriptional control of a galactose inducible promoter (*GAL1*).

The INVSc1 strain (Invitrogen) of yeast was transformed with the above recombinant constructs by the heat shock method (Elble[49], 1992) to assess the functionality of the genes and the derived proteins.

Enzyme Assay

Yeast cells containing chimeric GPAT genes were grown in SC-Ura (Bio 101) containing glucose. A 2.5 mL culture was initiated and grown for 18 hr at 28°C. A

fresh 10 mL culture was grown by adding equal number of yeast cells to this culture and grown for another 24 hr. GPAT gene expression was induced by transferring cells to growth medium containing galactose as follows : the cells were then transferred to 10 mL SC-Ura and galactose and the GPAT gene expression was induced for 24 hr. The yeast cells were in stationary phase by then. Cells were either used for protein extraction for enzyme assay or for lipid analysis.

Total proteins from the control and GPAT overexpressing cells were extracted by the glass bead or French Press method. and GPAT activity was assayed (Eccleston and Harwood[50], 1995). The products of the reaction were separated into LPA, PA, and DAG and TAG by thin layer chromatography (Zou et al.[51], 1999) and identified using standards. Total lipids in control and cells in which GPAT was expressed were measured by ^{1}H NMR (Rutar[52], 1989) and by gas liquid chromatography.

GPAT Activity in Yeast

All of the GPAT genes used produced functionally active protein when expressed by a galactose inducible promoter in yeast cells. Extracted GPATs were assayed for activity *in vitro*, by looking at the production of lysophosphatidic acid, and the results are listed in **Table 1**.

***TABLE 1: FORMATION OF LYSOPHOSPHATIDIC ACID BY ISOLATED GPATS EXPRESSED IN YEAST* IN VITRO**

Construct	substrate 18:1-CoA (pmol/min/mg)		Substrate 16:0-CoA (pmol/min/mg)	
	glucose	Galactose	Glucose	galactose
PYES2 (vector)	10.0	6.8	192	131
pYES2:*ctpgpat-tp*	9.6	33.0	229	175
pYES2:*ctpgpat-tp* *+errs*	13.7	44.4	138	157

PYES2: *pls*B	10.2	28.5	196	790
pYES2: *pls*B+*errs*	12.4	20.1	211	346

Increased GPAT activity lead to more total lipid production in the *in vitro* assay, as shown in **Table 2**.

Increased GPAT activity leads to enhanced lipid production *in vitro* and an increase in the proportions of all Kennedy pathway intermediates. The *pls*Bs were able to use both 16:0-CoA and 18:1-CoA, the former with several fold higher efficiency. The *ctpgpats* could use only 18:1-CoA but with greater efficiency than the *pls*Bs.

The transformants produce more lipids *in vivo*, relative to the control, as shown in **Tables 3 & 4**.

***TABLE 2: FORMATION OF TOTAL LIPIDS BY ISOLATED GPATS* IN VITRO**

Construct	substrate 18:1-CoA (pmol/min/mg)			Substrate 16:0-CoA (pmol/min/mg)		
	glucose	galactose	ratio*	glucose	galactose	ratio*
PYES2 (vector)	116	112	0.96	1140	513	0.44
pYES2:*ctpg pat-tp*	184	395	2.15	1170	868	0.74
pYES2:*ctpg pat-tp +errs*	175	341	1.95	717	771	1.07
PYES2: *pls*B	98	247	2.51	789	2990	3.77

Construct	substrate 18:1-CoA (pmol/min/mg)			Substrate 16:0-CoA (pmol/min/mg)		
	glucose	galactose	ratio*	glucose	galactose	ratio*
PYES2: *plsB+errs*	152	182	1.19	1250	1830	1.46

* galactose/glucose

TABLE 3: ESTIMATION OF OIL CONTENT IN CONTROL AND TRANSFORMED YEASTS BY ^{1}H NMR

Construct	^{1}H NMR response	Ratio of oil contents*	% increase
INVSc (wild type)	0.81	--	--
PYES2 (vector)	1.12	1.00	--
PYES2: *ctpgpat-tp*	1.3	1.17	17
PYES2: *ctpgpat-tp+errs*	1.68	1.51	51
PYES2: *plsB*	1.52	1.37	37
PYES2: *plsB+errs*	1.22	1.10	10

* cells grown in galactose/cells grown in glucose

TABLE 4: DETERMINATION OF OIL CONTENT OF WILD TYPE AND TRANSFORMED YEASTS BY GAS CHROMATOGRAPHY

construct	glucose (wt%)	Galactose (wt%)
INVSc (wild type)	4.4	4.4
PYES2 (vector	5.0	6.2
PYES2: *ctpgpat-tp*	5.5	8.8
PYES2: *ctpgpat-tp+errs*	6.0	7.4
plsB	7.5	7.6
plsB+errs	5.0	6.2

Legend to Tables 1 to 4: "glucose" and "galactose" indicate cells grown on glucose medium, and those grown on medium containing galactose (to induce the promoter), respectively.

N.B. comparison in **Table 4** can only be made within a construct, as the cells in different constructs may be at a different growth stage.

According to the process of the invention, lipid content is increased by enhancing GPAT activity. No manipulation of growth medium, growth conditions or substrates is required to achieve a higher lipid content.

Overexpression of GPATS having selectivity for specific fatty acids could be used to enhance the content of speciality oils in micro-organisms.

GPAT Activity in *A. thaliana*

For plant expression study, chimeric genes were cloned into the *Bam*HI site of the plant transformation vector, pHS737, under the control of a tandem 35S CaMV promoter with AMV translational enhancer and 35S polyA for constitutive expression. The recombinants were transferred into *Agrobacterium tumefaciens* GV 3101 for *Arabidopsis thaliana* transformation.

Arabidopsis plants were transformed by the floral dip method (Clough and Bent[53], 1998). Seeds (T_1) from these plants were collected and selected on a growth medium containing kanamycin. Transgenic plants were grown to maturity and seeds (T_2) from 10 individual plants were collected and used for lipid analysis. Wild type and plants transformed with vector alone were grown as controls along with the transformed plants.

Lipid Analysis

The fatty acid composition of seeds was determined by GC analysis following extraction of the oil and conversion of the triglycerides to fatty acid methyl esters. A known amount of C15 triglyceride was added to the seed sample as a tracer before oil extraction. Total seed lipid content was estimated on the basis of the recovery of C15 fatty acid methyl ester. C17:0 methyl ester was used as an internal standard for the chromatography. Fatty acid methyl esters were analysed using an HP 5850 gas chromatograph equipped with a DB-23 column (30m X 0.25mm; J & W Scientific, Folsom, CA). The GC conditions were: injector temperature and flame ionisation

detector temperature, 250°C. After an initial hold at 180°C for 1 min, the oven temperature was programmed to 240°C at 4°C/min and held at this temperature for 10 min.

Most of the transgenic plants appeared normal in morphology. The oil content and seed sizes of selected lines representing each construct are shown in **Table 5.**

TABLE 5: OIL CONTENT OF WILD TYPE AND TRANSFORMANT ARABIDOPSIS THALIANA SEEDS

Construct	transformant reference no.	Oil content		wt/100 seeds (mg)
		Wt%	% increase	
wild type	average (n=7)	26.9 ± 0.8	--	1.42 ± 0.29
pHS737 (vector)	average (n=7)	26.8 ± 1.09	--	1.41 ± 0.26
ctpgpat	315-2	2.4	20.9	2.47
	315-3	29.4	9.7	1.92
	315-4	30.1	12.3	1.90
	315-7	29.6	10.4	1.85
ctpgpat-tp	301-2	34.0	26.9	2.03
	301-3	34.6	29.1	1.90
	301-5	29.3	9.3	1.79
ctpgpat-tp +errs	302-2	29.4	9.7	1.44
	302-6	32.7	22.0	2.45
plsB	303-2	29.8	11.2	1.28
	303-3	30.8	14.9	1.98
	303-4	29.1	8.6	1.89

Construct	transformant reference no.	Oil content		wt/100 seeds (mg)
		Wt%	% increase	
	303-7	33.2	23.9	1.53
plsB+errs	304-1	30.9	15.2	1.51
	304-2	32.5	21.3	1.60
	304-3	30.3	13.1	1.38
	304-15	32.5	21.3	2.20

Seeds of plants transformed using only the pHS737 vector were indistinguishable in oil content from wild type control plants grown under the same conditions. All other gene constructs produced higher seed oil content. The unmodified *ctpgpat*, which would be expected to be expressed in the plastid, produced oil increases ranging from 10 to 21%. This suggests that LPA is released from the plastids and subsequently converted to TAGs. On average the greatest increase in oil was observed in seeds of transformants carrying the *ctpgpat-tp* gene (average +22%).

The *plsB* gene increased seed oil content by an average of 15%. The addition of an ER targeting sequence resulted in an average seed oil increase of 18%.

Seeds of plants transformed with the vector only did not differ significantly in average weight from wild type plants. Seeds of individual plants from each construct were significantly heavier than wild type and the pHS737 control; e.g. 315-2, 301-2, 302-6, 303-3 and 304-15. However, increased seed oil content was not always positively correlated with increased seed weight; e.g. 303-7 and 304-1.

Phenotypes presenting increased seed oil content and weight would result in increased yield from oilseed crops. Those presenting an increase in seed oil content without an increase in weight would provide more oil per tonne of seed, representing an additional advantage to oil seed producers.

Compositional changes were also observed in the seed oils of the transformants. Selected examples are illustrated in **Table 6.**

TABLE 6: FATTY ACID COMPOSITION OF WILD TYPE AND TRANSFORMED ARABIDOPSIS THALIANA

Construct/sample reference no.	wt% oil	18:1	18:3
wild type	26.9 ± 0.8 (n= 7)	14.4 ± 0.7	15.7 ± 0.8
PHS737 (vector)	26.8 ± 1.1 (n=6)	12.4 ± 1.3	16.9 ± 0.4
Ctpgpat			
315-2	32.4	13.6	18.4
315-4	30.1	14.7	16.5
315-6	25.2	9.1	18.4
315-8	27.1	9.2	19.9
315-10	28.3	12.4	17.5
ctpgpat-tp			
301-1	27.5	12.0	17.1
301-2	34.0	10.9	18.8
301-3	34.6	12.2	18.2
ctpgpat-tp+errs			
302-4	28.1	12.5	18.5
302-5	25.6	12.2	17.5
302-6	32.7	12.9	17.5
302-7	27.8	15.8	16.5
plsB			
303-1	28.4	12.0	17.8
303-3	30.8	11.4	17.6
303-7	29.1	14.7	17.1
	33.2	14.8	15.9

Construct/sample reference no.	wt% oil	18:1	18:3
plsB+errs			
304-2	32.5	12.4	17.0
304-3	30.3	12.1	16.8
304-4	23.5	10.7	18.0
304-9	27.4	13.9	17.4
304-15	32.5	14.1	16.9

Seed oils from plants transformed with the vector alone (*pHS737*) were not significantly different from the wild type.

The proportion of 18:1 Z9 (oleic acid) decreased and the proportion of 18:3 Z9, Z12, Z15 (α-linolenic acid) increased in some of the individual transformants of all contructs. Examples of this effect are 315-6, 315-8, 301-2, 302-5, 303-3 and 304-4.

INDUSTRIAL APPLICABILITY

The Kennedy pathway is common to all organisms. Transformation of yeast or plants with DNA encoding GPAT activity can be used both to enhance oil content, and to alter the fatty acid composition of TAGs. The use of GPATs with different acyl-CoA or acyl-ACP specificities can be used to tailor the fatty acid composition of the TAGs produced by the micro-organism or plant.

The method of the invention can manipulate oil synthesis in other organisms such as yeast, other fungae and algae for producing commodity and speciality oils. Increasing the oil content of feed quality grains would reduce the need for adding exogenous fats in the diets of animals and birds [54](Kishore and Shewmaker, 1999).

Safflower plastidial GPAT, the GPAT used in the examples, prefers unsaturated acyl-CoA or acyl-ACP, whereas *E. coli* GPAT, also used in the examples, prefers saturated acyl-CoA or acyl-ACP. These genes can be used to modify the type of fatty acid at the *sn*-1 position of TAGs. This enables the production of structured TAGs, in which the fatty acids occupying each position may be controlled. It is

believed that fatty acid absorption and physiological effect are related to TAG structure, and not just gross composition. This also has implications for manipulating fat content in humans and other animals.

DEPOSITS OF BIOLOGICAL MATERIAL

A plasmid library containing a mixture of plasmids (pYES2:*ctpgpat-tp*, pYES2:*ctpgpat-tp+errs*, pYES2:*plsB* and pYES2:*plsB+errs*) was deposited, according to the Budapest Treaty, on May 24, 2000, at the International Depository Authority of Canada (Winnipeg, Manitoba, Canada), under accession number **IDAC 240500-2** and reference pYEASTOIL.

A plasmid library containing a mixture of plasmids (pHS737:*ctpgpat*, pHS737:*ctpgpat-tp*, pHS737:*ctpgpat-tp+errs*, pHS737:*plsB* and pHS737:*plsB+errs*) was deposited according to the Budapest Treaty, on May 24, 2000, at the International Depository Authority of Canada (Winnipeg, Manitoba, Canada), under accession number **IDAC 240500-1** and reference pPLANTOIL.

SEQUENCES

SEQ ID NO: 1 is the DNA *ctpgpat* (encoding intact safflower plastidial GPAT)

SEQ ID NO: 2 is the DNA *ctpgpat-tp* (encoding safflower plastidial GPAT minus transit peptide)

SEQ ID NO: 3 is the DNA *ctpgpat-tp+errs* (encoding safflower plastidial GPAT minus transit peptide plus ER retention sequence)

SEQ ID NO: 4 is the DNA *plsB* (encoding *E. coli* GPAT)

SEQ ID NO: 5 is *plsB+errs* (encoding *E. coli* GPAT plus ER retention sequence)

SEQ ID NO: 6 is the protein encoded by *ctpgpat* (SEQ ID NO: 1; intact safflower plastidial GPAT)

SEQ ID NO: 7 is the protein encoded by *ctpgpat-tp* (SEQ ID NO: 2; safflower plastidial GPAT minus transit peptide)

SEQ ID NO: 8 is the protein encoded by *ctpgpat-tp+errs* (SEQ ID NO: 3; safflower plastidial GPAT minus transit peptide plus ER retention sequence)

SEQ ID NO: 9 is the protein encoded by *plsB* (SEQ ID NO: 4; *E. coli* GPAT)

SEQ ID NO: 10 is the protein encoded by *plsB+errs* (SEQ ID NO: 5; *E. coli* GPAT plus ER retention sequence)

International Depositary Authority of Canada

Room H5190,1015 Arlington Street, Tel: (204) 789-2002
Winnipeg, Manitoba, Canada R3E 3R2 Fax: (204) 789-2036

International Form IDAC/BP/4

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

(Issued pursuant to Rule 7.1 of the *Budapest Treaty* Regulations)

ATTACH COPIES OF THE ORIGINAL DEPOSIT CONTRACT AND VIABILITY STATEMENT

This International Depository Authority accepts the deposit of the microorganism specified below, which was received by it on May 24, 2000

To (Name of Depositor) Dr. S. L. MacKenzie

Address National Research Council of Canada, Plant Biotechnology Institute
110 Gymnasium Place, Saskatoon, Saskatchewan, S7N 0W9

IDENTIFICATION OF DEPOSIT

Reference assigned by depositor pPLANTOIL

Accession Number assigned by this IDA IDAC 240500-1

The deposit identified above was accompanied by:

☐ a scientific description (specify)_____

☐ a proposed taxonomic designation (specify)_____

Signature of person(s)authorized to represent IDAC:

_____

Date May 24, 2000

International Depositary Authority of Canada
Room H5190,1015 Arlington Street,
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International Form IDAC/BP/9

STATEMENT OF VIABILITY

(Issued pursuant to Rule 10.2 of the *Budapest Treaty* Regulations)

PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

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Address National Research Council of Canada, Plant Biotechnology Institute
110 Gymnasium Place, Saskatoon, Saskatchewan, S7N 0W9

DEPOSITOR

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IDENTIFICATION OF THE DEPOSIT

Accession Number given by the International Depository Authority IDAC 240500-1

Date of the original deposit (or most recent relevant date) May 24,2000

VIABILITY TEST

The viability of the deposit identified above was tested on (most recent test date) May 26, 2000

On the date indicated above, the culture was:

☒ viable

☐ no longer viable

Conditions under which the Viability Test were performed (to be filled in if the information has been requested and the results of the test were negative)

Signature of person(s) authorized to represent IDAC

Date May 29, 2000

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110 Gymnasium Place, Saskatoon, Saskatchewan, S7N 0W9

IDENTIFICATION OF DEPOSIT

Reference assigned by depositor pYEASTOIL

Accession Number assigned by this IDA IDAC 240500-2

The deposit identified above was accompanied by:

☐ a scientific description (specify)_____

☐ a proposed taxonomic designation (specify)_____

Signature of person(s)authorized to represent IDAC:

_____

Date May 24, 2000

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